DNA extraction of birch leaves by improved CTAB method and optimization of its ISSR system

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Abstract: The basic method of DNA extraction (CTAB) was improved as the multi-times STE-CTAB extraction method and used to extract the DNA of birch leaved in this experiment. Results showed that the improved method is suitable not only for genomic DNA extraction of birch but also for that of other plants. The purity of genomic DNA extracted by the multi-times STE-CTAB extraction method is higher than that by one time STE-CTAB method, and it does not need the process of RNase. The factors of influencing ISSR system were explored based on the genomic DNA of birch extracted by the two methods. The optimal conditions for ISSR system were determined as follows: Mg^{2+} concentration is 1.5–3.0 mmol·L^{-1}, dNTP concentration 0.10–0.25 mmol·L^{-1}, the quantity of Taq polymerase 0.5–2.0 U, template DNA 30–100 ng, and the concentration of primer is 0.2–0.4 μmmol·L^{-1}, and the reaction program was as: initial denaturation for 5 min at 94ºC, 30 cycles of denaturation for 30 s at 94ºC, annealing for 30 s at 51 ºC, extension for 30 s at 72ºC, and a final 7 min extension at 72 ºC.

Keywords: Birch; DNA; Extraction method; ISSR; Reaction system
CLC Number: S792.15 **Document code: A** **Article ID**: 1007-662X(2006)04-0298-03

Introduction

Betula, including more than 30 species, widespread distributes in almost all provinces of China. The complexity of genetic variation in *Betula* was mainly due to the variable climate and complicated geographic conditions in birch habitants (Jiang 2003).

Inter Simple Sequence Repeat (ISSR) is a kind of technology that uses SSR primers to amplify regions between simple repetitive DNA sequences. Since it was first reported by Zietkiewicz *et al*. (1994), this technology with great repeatability has been widely used in classification and systematic comparison of species, deduction of evolutionary relationship of species, and identification of varieties, and it also can be used to draw a genetic mapping (Tsumura *et al*. 1996).

The present study focuses on the extraction of high purity and quality of genomic DNA and the optimizing conditions of ISSR-PCR system of birch. Different extraction methods are used to the different plant materials that contain varied secondary metabolites. Since there is lots of saccharide in tissue cell of birch, the common used extraction methods are easy to cause the crossed contamination of samples by repetitious precipitate (Causse *et al*. 1994), consequently, low quality of DNA can affect the stability of PCR amplification reaction. Thus, we introduced the improved method about DNA extraction and optimization ISSR system of birch.

Materials and methods

Materials and reagents

Fresh leaves of birch were collected from the Experiment Forestry Farm of Northeast Forestry University, China, and stored at -70℃ for molecular analysis. CTAB extraction buffer: 2.0% (w/v) CTAB (Cetyltrimethylammonium bromide), NaCl 1.4 mol·L^{-1}, EDTA 20 mmol·L^{-1}, Tris-HCl 100 mmol·L^{-1} (pH 8.0), 2 mL·L^{-1} of ß-mercaptoethanol; Washing buffer: STE (NaCl 1.4 mol·L^{-1}, EDTA 20 mmol·L^{-1}, Tris-HCl 100 mmol·L^{-1}), chloroform, anhydrous alcohol and 70% alcohol, TE (10 mmol·L^{-1} Tris-HCl, pH 7.4, EDTA 1 mmol·L^{-1}). The sequences of ISSR primers were provided by Columbia University, Canada, and synthesized by TaKaRa Company.

Genomic DNA extraction

(1) Approximately 0.5 g of fresh leaves was put into a pre-chilled mortar and ground into powder in liquid nitrogen.

(2) The powder was transferred into a 1.5-mLcentrifuge tube quickly, added with 800 μL STE and 80 μL ß-mercaptoethanol, and completely agitated. Centrifuging was conducted at 5 000 r·min^{-1} for 3 or 5 min at room temperature. After pouring off supernatant, 800-μL STE was added into centrifuge tube and completely agitated again, then centrifuged at 5 000 r·min^{-1} for 3 or 5 min.

(3) CTAB buffer (600 μL) was added into the tube, mixed by shaking gently for about 10 min. Then, the mixture was incubated at 65ºC for 30 min, inverted every 5–10 min, and finally cooled to room temperature.

(4) 600 μL chloroform was added into the tube, shaking gently about 2–3 min, and centrifuged at 13 000 r·min^{-1} for 5 min.

(5) After pouring off the supernatant, 600 μL CTAB buffer were added into the tube and centrifuged at 13 000 r·min^{-1} for 5 min, then, the supernatant was withdrawn, 600 μL chloroform were added, and centrifuged at 13000 r·min^{-1} for 5 min, after then, retaining precipitation and adding 600 μL CTAB buffer again, centrifuging at 13 000 r·min^{-1} for 5 min.

(6) The (4) step was repeated 3 times.

(7) After the last centrifuging, the retained supernatant was added into ethanol. The tube was stored at –20 ºC for at least 30 min, and centrifuged at 12 000 r·min^{-1} for 2 min.

(8) The precipitated DNA was washed twice with 70% alcohol and centrifuged. The DNA pellet was air-dried, and added about

Foundatio project: This paper was supported by National Natural Science Foundation of China (No. 30571513) and National High Technology Research and Development Program of China (863 Program) (No. 2002AA241080).
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Received date: 2006-03-22
Accepted date: 2006-04-11
Responsible editor: Song Funan

50 μL TE buffer for a short-term DNA storage.

This method was called multi-times STE-CTAB. The purity and quality of genomic DNA were determined by UV-spectrophotometer and agarose gels electrophoresis.

ISSR-PCR reaction system

ISSR-PCR amplification of the sample was performed in Perkin-Elmer 9700 Thermal Cycler, according to the following reaction system. Initial denaturation was for 5 min at 94 ºC, followed by 30 cycles of denaturation for 30 s at 94 ºC, annealing for 30 s at 51 ºC, extension for 30 s at 72 ºC, and a final 7 min extension at 72 ºC (Yang *et al*. 2005). The amplified products were segregated in 1.5% agarose gels (Containing EB 0.5 $\mu g \cdot mL^{-1}$). After electrophoresis, gels were put into UVP Gel Documentation Systems (GDS7600) for observing and taking photos.

Results

UV spectrophotometric evaluation of DNA

Ultraviolet absorption checking: the genomic DNA was diluted with TE buffer, the OD value (A_{260}/ A_{280}) and DNA concentration were measured, and the control was TE buffer. When the ratio of A_{260}/ A_{280} is between 1.8–1.9, it shows that protein is removed completely (Nagaoka *et al*. 1997), and DNA purity and content are very high. If the ratio of A_{260}/ A_{280} is low, and DNA has been polluted seriously by protein or polysaccharide (Table 1).

Table 1. OD values **(A_{260}/ A_{280}) of birch DNA**

Methods	A_{260}	A_{280}	A_{260}/ A_{280}	DNA content /$\mu g \cdot mL^{-1}$
One-CTAB	0.069	0.051	1.352	680
	0.043	0.033	1.287	430
Multi-CTAB	0.097	0.052	1.874	970
	0.092	0.049	1.861	920

Agarose gel electrophoresis testing

Electrophoresis of extracted DNA was performed in 0.8% agarose gels (Fig.1 A, B). The result indicated that DNA extracted by the method of multi-times STE-CTAB was more distinct and purer, and no RNA, compared with that extracted by one time STE-CTAB.

Influence factors of ISSR-PCR reactions

(1) Content of template DNA: Many studies showed that 5–500 ng template DNA could offer an amplification result (Huang *et al*. 2000). The optimal content of DNA used for ISSR-PCR mainly depends on the kind of material and purity of template DNA. In this experiment, 5, 9, 15, 30, 45, 60, 75, 90, 120, 150 and 200 ng of template DNA was separately used to conduct the ISSR-PCR. If template content was higher than 120 ng, many amplified fragments would be so light, and the template DNA content was lower than 30 ng, amplified fragments were not apparent or lost. The result showed that 30–100 ng of template DNA was better for ISSR-PCR (Fig.2).

(2) Concentration of primer: 0.10, 0.15, 0.20, 0.25, 0.30, 0.40 and 0.60 $\mu mol \cdot L^{-1}$ of primer in 20 μL total volume of reaction was used for ISSR. Lower prime concentration (<0.20 $\mu mol \cdot L^{-1}$) can not obtain amplification products, and higher concentration (>0.40 $\mu mol \cdot L^{-1}$) may produce new fragments. The result showed that 0.2–0.4 $\mu mol \cdot L^{-1}$ concentration of primer was better for ISSR system. (Fig.3)

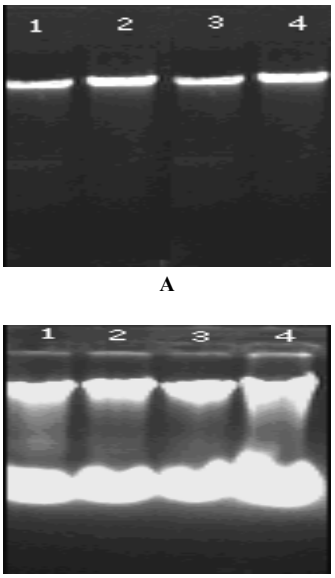

Fig. 1 A: DNA extraction of multi-times STE-CTAB; B: DNA extraction of one time STE-CTAB. Note: Lane 1–4: bands of birch DNA

Fig. 2 The amplified results of different template DNA concentrations. Note: Lane 1–11 DNA concentration: 5, 9, 15, 30, 45, 60, 75, 90, 120, 150, 200 ng.

Fig. 3 The ISSR amplified results of different primer concentrations
Note: Lane 1–7 are the primer concentrations of 0.60, 0.40, 0.30, 0.25, 0.20, 0.15, 0.10 $\mu mol \cdot L^{-1}$, respectively.

(3) Mg^{2+} concentration: Mg^{2+} was an important factor in stimulating Taq DNA polymerase, opening double chains of prime and template DNA, and annealing temperature, so the appropriate Mg^{2+} concentration was essential to the ISSR. The Mg^{2+} concentrations of 0.2, 0.5, 1.0, 2.0, 2.5 and 3.0 $mmol \cdot L^{-1}$ were separately used to perform the ISSR system. Usually, Mg^{2+} concentration was between 0.5–2.5 $mmol \cdot L^{-1}$, but the best Mg^{2+} concentration for ISSR was 1.5–3.0 $mmol \cdot L^{-1}$. (Fig.4)

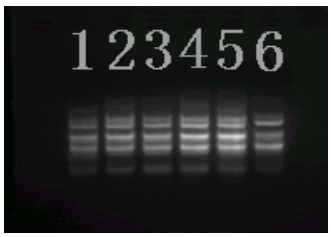

Fig. 4 The ISSR amplified results of different Mg^{2+} concentrations
Note: Lane 1–6 are the Mg^{2+} concentrations of 0.2, 0.5, 1.0, 2.0, 2.5, 3.0 mmol·L^{-1}, respectively.

(4) Concentration of dNTP: dNTP was an important material for PCR, and its concentration in reaction was usually 50–200 μmol·L^{-1}. The dNTP concentrations of 50, 100, 150, 200, 250, 300, 400 μmol·L^{-1} were separately used in this experiment. When the concentration was too high (> 300 μmol·L^{-1}), it may result in the wrong amplification, on the contrary, it may produce few fragments. The optimal concentration of dNTP was between 100–300 μmol·L^{-1} for ISSR of birch (Fig.5).

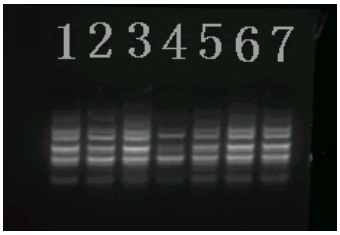

Fig. 5 The ISSR amplified results of different dNTP concentration
Note: Lane 1–7 are the dNTP concentrations of 50, 100, 150, 200, 250, 300, 400 μmol·L^{-1}, respectively.

ISSR-PCR reaction system

From above experiment, we can conclude that the best reaction system is determined as 2.5 mmol·L^{-1} Mg^{2+}, 10 mmol·L^{-1} dNTP, 10 mmol·L^{-1} primer, 1×Taq DNA polymerase buffer, and 45 ng template DNA in a total volume of 20 μL. The amplification conditions of birch ISSR-PCR is as follows: Initial denaturation for 5 min at 94 ºC, then denaturation for 30 s at 94 ºC, by 30 cycles, annealing for 30 s at 51 ºC, extension for 30 s at 72 ºC, and a final 7 min extension at 72 ºC, (Yang *et al.* 2005). The PCR products were isolated by electrophoresis and photographed with UVP Gel Documentation Systems (GDS7600). Molecular weight was estimated by using a 100 bp DNA ladder (MBI).

Discussion

From the amplification result, the extraction method of multi-times STE-CTAB was easier and faster, the amplifying result was more distinct, and DNA purity was higher. Extraction method of one time STE-CTAB had worse stability and repeatability. The DNA extracted by multi-times STE-CTAB was completely suitable for molecular biology research, and the result of ISSR amplification was stable.

In general, the purity of genomic DNA extracted by the multi-times STE-CTAB extraction method is higher than that by one time STE-CTAB. And dilution of the DNA was sufficient for successful PCR. The resultant DNA was suitable for molecular analyses of microbial communities in biological treatment system.

For the extraction process, we should pay attention to two steps: one is that the sample should be treated all in liquid nitrogen, and ground into powder; the other, in order to obtain the perfection of DNA fragments, every step of extraction should avoid long-time and violent agitation.

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